## Plasmalogen biosynthesis by anaerobic bacteria: Identification of a two-gene operon responsible for plasmalogen production in *Clostridium perfringens*

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<b>Bacterial Strains</b>	Description	Source
C. perfringens HN13	Wild-type C. perfringens strain	Nariya et. al., 2011 (reference 15)
<i>C. perfringens</i> HN13 Tn library	Previously constructed Tn mutant library	Prof. Stephen Melville
C. perfringens HN13-∆PlsA PlsR	C. perfringens HN13 lacking CPE1194/1195	This work
E. coli BL21(DE3)	<i>E. coli</i> strain for overexpression of pPIsCP	New England Biolabs
<i>E. coli</i> DH5α	E. coli strain for cloning	New England Biolabs
<i>E. faecalis</i> OG1RF	<i>E. faecalis</i> model strain analyzed for plasmalogen content	A gift from Prof. Danielle Garsin
Plasmids	Description	Source
pUC19	Plasmid for capturing Tn insertion sites fragments	New England Biolabs
pCM-GalK	Plasmid to create deletions in <i>C.</i> perfringens HN13	Nariya et. al., 2011 (reference 15)
pCM-GALK-⊿1194/1195	pCM-GALK containing 5' and 3' flanking regions of CPE1194/CPE1195	This work
pET-28a	inducible E. coli expression vector	Novagen
pPIsCP	pET-28a containing <i>CPE1194/1195</i> inserted at Nhel/BamHI	This work
pPIsEF	pET-29b containing the <i>E. faecalis</i> EF1327 gene inserted at Ndel/Xhol	This work
Dulus aus	Description	0
Primers	Description	Sequence
OHL21	sequencing primer to map cloned Tn insertion sites	GCAATGAAACACGCCAAAGTAAACAATTTAAGTACCG
OHL22	sequencing primer to map cloned Tn insertion sites	GTTTTATTATTTGGTTGAGTACTTTTTCACTCG
DJ031	forward primer for N-terminal CPE1194/1195 flanking region with a BamHI site	AAAggatccCAAGTCATCCAGGATGCAAG
DJ032	reverse primer for N-terminal CPE1194/1195 flanking region	CCATAATTCCTCTAATCATACCCCCTTTTATATTTTCTAGTTC
DJ033	forward primer for C-terminal CPE1194/1195 flanking region	GAACTAGAAAATATAAAAGGGGGTATGATTAGAGGAATTATGG
DJ034	reverse primer for C-terminal CPE1194/1195 flanking region with a Nhel site	AAAgctagcTTGATACCTTATATCTGTTTTTTCCTAGA
DJ049	forward sequencing primer for CPE1194/1195 deletion in C. perfringens HN13	GGCTTCTAATCCATTTACAGCCTTAAATACAGTGTAC
DJ050	reverse sequencing primer for <i>CPE1194/1195</i> deletion in <i>C.</i> perfringens HN13	GAGGATTTGATCAAAAGAGCCAAAGAGGG
DJ009	forward primer containing Nhel for Pls ORF (CPE1194/1195)	CATGgctagcATGTATTACAAAATAGGTATTGATG
DJ010	forward primer containing Xhol for Pls ORF ( <i>CPE1194/1195</i> )	CTctcgagTCATTCCTGTGCTATAG

 Table S1. Bacterial strains, plasmids, and primers used in this study.

		activation				% ident	ity to:
		Subunit/				PlsA	PlsA
Organism	Enzyme type	domain	Mass (kDa)	Module	NCBI accession	(Act1)	(Act2)
Clostridium perfringens	plasmalogen synthase (CPE1195 1-288)	PlsA (Act1)	32	activation	WP 025647548	100.0	30.8
Clostridium perfringens	plasmalogen synthase (CPE1195 289-620)	PlsA (Act2)	37	activation	WP 025647548	30.8	100.0
Rhodopseudomonas palustris	benzovl-CoA reductase	BcrA	48	activation	WP 013503789	22.9	26.0
Rhodopseudomonas palustris	benzovl-CoA reductase	BcrD	29	activation	WP_013503788	23.4	21.5
Thauera aromatica	benzovl-CoA reductase	BcrA	48	activation	WP 107220519	22.0	25.2
Thauera aromatica	benzovl-CoA reductase	BcrD	30	activation	WP 107220520	21.9	22.3
Azoarcus sp. CIB	benzovl-CoA reductase	BzdP	28	activation	WP 050415405	24.1	23.8
Azoarcus sp. CIB	benzovI-CoA reductase	BzdQ	33	activation	WP 050415406	23.6	25.1
Ferroglobus placidus	benzovl-CoA reductase	BzdP	28	activation	WP 012965687	27.1	21.2
Ferroglobus placidus	benzovl-CoA reductase	BzdQ	33	activation	WP_012965688	27.8	28.7
Fusobacterium nucleatum	(R)-2-bydroxydutaryl-CoA debydratase	HadC	27	activation	WP_008694125	25.0	27.6
Acidimonacoccus fermentans	(R)-2-hydroxyglutaryl-CoA dehydratase	HadC	27	activation	WP_012939153	29.8	28.4
Clostridium difficile	(R)-2-hydroxyisocaprovl-CoA debydratase	Hadl	28	activation	WP 009888223	26.5	31.0
	(R)-2-lactov/-CoA dehydratase		20	activation	WP_066048110	28.0	28.7
	(R)-phenyllactyl-CoA dehydratase	Fidi	28	activation	WP 003492348	27.6	30.0
Archaeoglobus fulgidus	(R)-phonyllactyl-CoA dehydratase	HadC	20	activation	WP_003432340	27.0	30.0
Archaeoglobus luigidus	(N)-prienyllactyl-00A denydratase	riguo			<u> </u>	21.0	50.0
		roduction/					
		debudretiien				0/ ident	itu ta i
							ly lo:
	<b>F</b>	Subunit				PISA	PISK
Organism	Enzyme type	domain	Mass (kDa)	Module	NCBI accession	(Red1)	(Red2)
Clostridium perfringens	In lasmalogen synthese (CPE1195621-975)					1 100 0	
01 11 11 11			41		WP_025647548	100.0	19.8
Clostridium perfringens	plasmalogen synthase (CPE1194)	PlsR (Red2)	41	reduction/dehydration	WP_025647548 WP_025647548	100.0 19.8	19.8
Clostridium perfringens Rhodopseudomonas palustris	plasmalogen synthase (CPE1194) benzoyl-CoA reductase	PlsR (Red2) BcrC	41 48 44	reduction/dehydration reduction	WP_025647548 WP_025647548 WP_013503791	100.0 19.8 19.9	19.8 100.0 18.3
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase	PISR (Red2) BcrC BcrB	41 48 44 50	reduction/dehydration reduction reduction	WP_025647548 WP_025647548 WP_013503791 WP_013503790	100.0 19.8 19.9 20.9	100.0 18.3 23.1
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase	PlsR (Red2) BcrC BcrB BcrC	41 48 44 50 44	reduction/dehydration reduction reduction reduction reduction	WP_025647548 WP_025647548 WP_013503791 WP_013503790 WP_107220517	100.0 19.8 19.9 20.9 19.4	19.8 100.0 18.3 23.1 20.5
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase	PISR (Red2) BcrC BcrB BcrC BcrB BcrB	41 48 44 50 44 49	reduction/denydration reduction reduction reduction reduction reduction	WP_025647548 WP_025647548 WP_013503791 WP_013503790 WP_107220517 WP_107220518	100.0 19.8 19.9 20.9 19.4 19.8	19.8 100.0 18.3 23.1 20.5 21.5
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase	PISR (Red2) BcrC BcrB BcrC BcrB BcrB BzdN	41 48 44 50 44 49 43	reduction/denydration reduction reduction reduction reduction reduction reduction	WP_025647548 WP_025647548 WP_013503791 WP_013503790 WP_107220517 WP_107220518 WP_050415404	100.0           19.8           19.9           20.9           19.4           19.8           21.6	19.8         100.0         18.3         23.1         20.5         21.5         18.9
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB Azoarcus sp. CIB	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase	PIsR (Red2) PIsR (Red2) BcrC BcrB BcrC BcrB BzdN BzdO	41 48 44 50 44 49 43 51	reduction/dehydration reduction reduction reduction reduction reduction reduction	WP_025647548 WP_025647548 WP_013503791 WP_013503790 WP_107220517 WP_107220518 WP_050415404 WP_050418279	100.0           19.8           19.9           20.9           19.4           19.8           21.6           17.7	19.8         100.0         18.3         23.1         20.5         21.5         18.9         17.3
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB Azoarcus sp. CIB Ferroglobus placidus	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase	PISR (Red2) PISR (Red2) BcrC BcrB BcrC BcrB BzdN BzdO BzdN	41 48 44 50 44 49 43 51 45	reduction/dehydration reduction reduction reduction reduction reduction reduction reduction	WP_025647548 WP_025647548 WP_013503791 WP_013503790 WP_107220517 WP_107220518 WP_050415404 WP_050418279 WP_148212195	100.0           19.8           19.9           20.9           19.4           19.8           21.6           17.7           21.0	19.8         100.0         18.3         23.1         20.5         21.5         18.9         17.3         20.1
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB Azoarcus sp. CIB Ferroglobus placidus Ferroglobus placidus	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase	PlsR (Red2) BcrC BcrB BcrB BcrB BcrB BcdN BzdO BzdN BzdO	41 48 44 50 44 49 43 51 45 49	reduction/dehydration reduction reduction reduction reduction reduction reduction reduction reduction	WP_025647548 WP_025647548 WP_013503791 WP_013503790 WP_107220517 WP_107220518 WP_050415404 WP_050418279 WP_148212195 WP_048086821	100.0           19.8           19.9           20.9           19.4           19.8           21.6           17.7           21.0           20.1	19.8         100.0         18.3         23.1         20.5         21.5         18.9         17.3         20.1         19.3
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB Azoarcus sp. CIB Ferroglobus placidus Ferroglobus placidus Fusobacterium nucleatum	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase	PIsR (Red2) BcrC BcrB BcrB BcrC BcrB BzdN BzdO BzdN BzdO HgdA	41 48 44 50 44 49 43 51 45 49 50	reduction/dehydration reduction reduction reduction reduction reduction reduction reduction reduction reduction dehydration	WP_025647548 WP_025647548 WP_013503791 WP_013503790 WP_107220517 WP_107220518 WP_050415404 WP_050418279 WP_148212195 WP_048086821 WP_005909813	100.0           19.8           19.9           20.9           19.4           19.8           21.6           17.7           21.0           20.1	19.8         100.0         18.3         23.1         20.5         21.5         18.9         17.3         20.1         19.3         19.9
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB Azoarcus sp. CIB Ferroglobus placidus Ferroglobus placidus Fusobacterium nucleatum Fusobacterium nucleatum	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase	PIsR (Red2) BcrC BcrB BcrB BcrC BcrB BzdN BzdO BzdN BzdO HgdA HgdB	41 48 44 50 44 49 43 51 45 49 50 42	reduction/dehydration reduction/dehydration reduction reduction reduction reduction reduction reduction reduction dehydration	WP_025647548 WP_025647548 WP_013503791 WP_013503790 WP_107220517 WP_107220518 WP_050415404 WP_050418279 WP_148212195 WP_048086821 WP_005909813 WP_005909812	100.0 19.8 19.9 20.9 19.4 19.8 21.6 17.7 21.0 20.1 19.3 22.9	19.8           100.0           18.3           23.1           20.5           21.5           18.9           17.3           20.1           19.3           19.3           19.3
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB Azoarcus sp. CIB Ferroglobus placidus Ferroglobus placidus Fusobacterium nucleatum Fusobacterium nucleatum Acidimonacoccus fermentans	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase	PIsR (Red2) BcrC BcrB BcrB BcrC BcrB BzdN BzdO BzdN BzdO HgdA HgdA	41 48 44 50 44 49 43 51 45 49 50 42 54	reduction/dehydration reduction/dehydration reduction reduction reduction reduction reduction reduction reduction dehydration dehydration	WP_025647548 WP_025647548 WP_013503791 WP_013503790 WP_107220517 WP_107220518 WP_050415404 WP_050418279 WP_148212195 WP_048086821 WP_005909813 WP_005909812 WP_012939152	100.0           19.8           19.9           20.9           19.4           19.8           21.6           17.7           21.0           20.1           19.3           22.9           21.0	19.8           100.0           18.3           23.1           20.5           21.5           18.9           17.3           20.1           19.3           19.3           19.3           19.3           19.3
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB Azoarcus sp. CIB Ferroglobus placidus Ferroglobus placidus Fusobacterium nucleatum Fusobacterium nucleatum Acidimonacoccus fermentans Acidimonacoccus fermentans	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase	PlsR (Red2) BcrC BcrB BcrB BcrB BzdN BzdO BzdN BzdO HgdA HgdA HgdB	$ \begin{array}{r} 41\\ 48\\ 44\\ 50\\ 44\\ 49\\ 43\\ 51\\ 45\\ 49\\ 50\\ 42\\ 54\\ 42\\ 54\\ 42\\ \end{array} $	reduction/dehydration reduction/dehydration reduction reduction reduction reduction reduction reduction reduction dehydration dehydration dehydration	WP_025647548 WP_025647548 WP_013503791 WP_013503790 WP_107220517 WP_107220518 WP_050415404 WP_050418279 WP_050418279 WP_048086821 WP_048086821 WP_005909813 WP_005909812 WP_012939152	100.0           19.8           19.9           20.9           19.4           19.8           21.6           17.7           21.0           20.1           19.3           22.9           21.0           21.0	19.8           100.0           18.3           23.1           20.5           21.5           18.9           17.3           20.1           19.3           19.9           21.5           18.9           17.3           20.1           19.3           19.9           21.5           18.0           20.1
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB Azoarcus sp. CIB Ferroglobus placidus Ferroglobus placidus Fusobacterium nucleatum Acidimonacoccus fermentans Acidimonacoccus fermentans Clostridium difficile	plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase	PlsR (Red2) BcrC BcrB BcrB BcrB BzdN BzdO BzdN BzdO HgdA HgdA HgdB HgdB HadB	$ \begin{array}{r} 41\\ 48\\ 44\\ 50\\ 44\\ 49\\ 43\\ 51\\ 45\\ 49\\ 50\\ 42\\ 54\\ 42\\ 46\\ \end{array} $	reduction/dehydration reduction/dehydration reduction reduction reduction reduction reduction reduction reduction dehydration dehydration dehydration dehydration	WP_025647548 WP_025647548 WP_013503790 WP_013503790 WP_107220517 WP_107220518 WP_050415404 WP_050418279 WP_048086821 WP_048086821 WP_005909813 WP_005909812 WP_012939152 WP_012939151 WP_009888224	100.0 19.8 19.9 20.9 19.4 19.8 21.6 17.7 21.0 20.1 19.3 22.9 21.0 21.5 22.0	19.8           100.0           18.3           23.1           20.5           18.9           17.3           20.1           19.3           19.9           21.5           18.9           17.3           20.1           19.3           19.9           21.5           18.0           20.1           12.5           18.0           20.1           21.9
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB Azoarcus sp. CIB Ferroglobus placidus Ferroglobus placidus Fusobacterium nucleatum Fusobacterium nucleatum Acidimonacoccus fermentans Acidimonacoccus fermentans Clostridium difficile	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxysocaproyl-CoA dehydratase (R)-2-hydroxyisocaproyl-CoA dehydratase (R)-2-hydroxyisocaproyl-CoA dehydratase	PlsR (Red1) PlsR (Red2) BcrC BcrB BcrC BcrB BzdN BzdO BzdN BzdO HgdA HgdB HgdB HgdB HadB HadB HadC	$ \begin{array}{r} 41\\ 48\\ 44\\ 50\\ 44\\ 49\\ 43\\ 51\\ 45\\ 49\\ 50\\ 42\\ 54\\ 42\\ 46\\ 42\\ \end{array} $	reduction/dehydration reduction/dehydration reduction reduction reduction reduction reduction reduction reduction dehydration dehydration dehydration dehydration dehydration dehydration	WP_025647548 WP_025647548 WP_013503790 WP_107220517 WP_107220518 WP_050415404 WP_050418279 WP_148212195 WP_048086821 WP_005909813 WP_005909812 WP_012939152 WP_012939151 WP_009888224 WP_009888224	100.0           19.8           19.9           20.9           19.4           19.8           21.6           17.7           21.0           20.1           19.3           22.9           21.0           21.5           22.0           20.1	19.8           100.0           18.3           23.1           20.5           21.5           18.9           17.3           20.1           19.3           19.9           21.5           18.0           20.1           21.5           18.0           20.1           21.5
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB Azoarcus sp. CIB Ferroglobus placidus Ferroglobus placidus Fusobacterium nucleatum Fusobacterium nucleatum Acidimonacoccus fermentans Clostridium difficile Clostridium proprionicum	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxysocaproyl-CoA dehydratase (R)-2-hydroxysocaproyl-CoA dehydratase (R)-2-hydroxysocaproyl-CoA dehydratase (R)-2-hydroxysocaproyl-CoA dehydratase (R)-2-lactoyl-CoA dehydratase	PlsR (Red2) PlsR (Red2) BcrC BcrB BcrC BcrB BzdN BzdO BzdN BzdO HgdA HgdA HgdA HgdB HadB HadB HadC LcdA	$ \begin{array}{r} 41\\ 48\\ 44\\ 50\\ 44\\ 49\\ 43\\ 51\\ 45\\ 49\\ 50\\ 42\\ 54\\ 42\\ 46\\ 42\\ 46\\ 42\\ 47\\ \end{array} $	reduction/dehydration reduction reduction reduction reduction reduction reduction reduction reduction dehydration dehydration dehydration dehydration dehydration dehydration dehydration	WP_025647548 WP_025647548 WP_013503790 WP_107220517 WP_107220518 WP_050415404 WP_050418279 WP_148212195 WP_04806821 WP_005909813 WP_005909813 WP_005909812 WP_012939151 WP_009888224 WP_009888224 WP_009888224	100.0           19.8           19.9           20.9           19.4           19.8           21.6           17.7           21.0           20.1           19.3           22.9           21.0           20.1           19.3           22.9           21.0           20.0           21.5           22.0           20.0           19.2	19.8           100.0           18.3           23.1           20.5           21.5           18.9           17.3           20.1           19.3           19.3           20.1           19.3           20.1           19.3           20.1           19.3           20.1           19.3           19.9           21.5           18.0           20.1           21.9           22.7           22.1
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB Azoarcus sp. CIB Ferroglobus placidus Ferroglobus placidus Fusobacterium nucleatum Acidimonacoccus fermentans Acidimonacoccus fermentans Clostridium difficile Clostridium difficile Clostridium proprionicum	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxysocaproyl-CoA dehydratase (R)-2-hydroxysocaproyl-CoA dehydratase (R)-2-hydroxyisocaproyl-CoA dehydratase (R)-2-lactoyl-CoA dehydratase (R)-2-lactoyl-CoA dehydratase	PISR (Red1) PISR (Red2) BcrC BcrB BcrC BcrB BzdN BzdO BzdN BzdO HgdA HgdA HgdA HgdB HadB HadB HadC LcdA LcdB	$ \begin{array}{r} 41\\ 48\\ 44\\ 50\\ 44\\ 49\\ 43\\ 51\\ 45\\ 49\\ 50\\ 42\\ 54\\ 42\\ 46\\ 42\\ 46\\ 42\\ 47\\ 42\\ 42\\ 47\\ 42\\ 42\\ 42\\ 42\\ 44\\ 42\\ 44\\ 42\\ 44\\ 42\\ 44\\ 42\\ 44\\ 42\\ 44\\ 44$	reduction/dehydration reduction reduction reduction reduction reduction reduction reduction reduction dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration	WP_025647548 WP_025647548 WP_013503791 WP_013503790 WP_107220517 WP_107220517 WP_050415404 WP_050418279 WP_148212195 WP_048086821 WP_048086821 WP_005909813 WP_005909812 WP_012939155 WP_012939151 WP_009888224 WP_009888224 WP_0066048111 WP_066048114	100.0           19.8           19.9           20.9           19.4           19.8           21.6           17.7           21.0           20.1           19.3           22.9           21.0           21.0           20.1           19.3           22.9           21.0           21.5           22.0           20.0           19.2           19.4	19.8           100.0           18.3           23.1           20.5           21.5           18.9           17.3           20.1           19.3           19.9           21.5           18.9           17.3           20.1           19.3           19.9           21.5           18.0           20.1           21.9           22.7           22.1           23.5
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB Azoarcus sp. CIB Ferroglobus placidus Ferroglobus placidus Fusobacterium nucleatum Acidimonacoccus fermentans Acidimonacoccus fermentans Clostridium difficile Clostridium difficile Clostridium proprionicum Clostridium proprionicum	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxysocaproyl-CoA dehydratase (R)-2-hydroxysocaproyl-CoA dehydratase (R)-2-hydroxysocaproyl-CoA dehydratase (R)-2-lactoyl-CoA dehydratase (R)-2-lactoyl-CoA dehydratase (R)-2-lactoyl-CoA dehydratase (R)-2-lactoyl-CoA dehydratase	PlsR (Red1) PlsR (Red2) BcrC BcrB BcrC BcrB BzdN BzdO BzdN BzdO HgdA HgdA HgdB HadB HadB HadB LcdA LcdB FldB	$ \begin{array}{r} 41\\ 48\\ 44\\ 50\\ 44\\ 49\\ 43\\ 51\\ 45\\ 49\\ 50\\ 42\\ 54\\ 42\\ 46\\ 42\\ 46\\ 42\\ 47\\ 42\\ 46\\ 46\\ 42\\ 46\\ 46\\ 42\\ 46\\ 46\\ 46\\ 42\\ 46\\ 46\\ 46\\ 46\\ 46\\ 46\\ 46\\ 46\\ 46\\ 46$	reduction/dehydration reduction reduction reduction reduction reduction reduction reduction reduction dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration	WP 025647548 WP 025647548 WP 013503791 WP 013503790 WP 107220517 WP 107220517 WP 050415404 WP 050418279 WP 148212195 WP 048086821 WP 005909813 WP 005909813 WP 005909812 WP 012939151 WP 009888224 WP 009888224 WP 009888224 WP 006048111 WP 066048114 WP 003492352	100.0 19.8 19.9 20.9 19.4 19.8 21.6 17.7 21.0 20.1 19.3 22.9 21.0 21.5 22.0 20.0 21.5 22.0 20.0 19.2 19.4 23.5	19.8           100.0           18.3           23.1           20.5           21.5           18.9           17.3           20.1           19.3           19.9           21.5           18.0           20.1           19.3           19.9           21.5           18.0           20.1           22.5           20.1           21.9           22.7           22.7           22.1           23.5           20.8
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB Azoarcus sp. CIB Ferroglobus placidus Ferroglobus placidus Ferroglobus placidus Fusobacterium nucleatum Acidimonacoccus fermentans Acidimonacoccus fermentans Clostridium difficile Clostridium difficile Clostridium proprionicum Clostridium proprionicum Clostridium sporogenes	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxysiocaproyl-CoA dehydratase (R)-2-lactoyl-CoA dehydratase (R)-2-lactoyl-CoA dehydratase (R)-2-lactoyl-CoA dehydratase (R)-phenyllactyl-CoA dehydratase (R)-phenyllactyl-CoA dehydratase	PlsR (Red1) PlsR (Red2) BcrC BcrB BcrB BcrC BcrB BzdN BzdO BzdN BzdO HgdA HgdB HgdA HgdB HadB HadB HadB LcdA LcdB FldB FldC	$ \begin{array}{r} 41\\ 48\\ 44\\ 50\\ 44\\ 49\\ 43\\ 51\\ 45\\ 49\\ 50\\ 42\\ 54\\ 42\\ 46\\ 42\\ 46\\ 42\\ 46\\ 42\\ 46\\ 43\\ \end{array} $	reduction/dehydration reduction reduction reduction reduction reduction reduction reduction reduction dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration	WP 025647548 WP 025647548 WP 013503791 WP 013503790 WP 107220517 WP 050415404 WP 050415404 WP 050418279 WP 148212195 WP 048086821 WP 005909813 WP 005909812 WP 012939151 WP 009888224 WP 009888224 WP 009888224 WP 009888224 WP 006048111 WP 006048114 WP 003492352 WP 003492354	100.0 19.8 19.9 20.9 19.4 19.8 21.6 17.7 21.0 20.1 19.3 22.9 21.0 21.5 22.0 20.0 19.4 23.5 20.0	19.8           100.0           18.3           23.1           20.5           21.5           18.9           17.3           20.1           19.3           19.9           21.5           18.0           20.1           19.3           19.9           21.5           18.0           20.1           21.5           18.0           20.1           21.9           22.7           22.7           22.7           22.7           22.7           22.7           22.7           20.8           24.3
Clostridium perfringens Rhodopseudomonas palustris Rhodopseudomonas palustris Thauera aromatica Thauera aromatica Azoarcus sp. CIB Azoarcus sp. CIB Ferroglobus placidus Ferroglobus placidus Fusobacterium nucleatum Fusobacterium nucleatum Acidimonacoccus fermentans Acidimonacoccus fermentans Clostridium difficile Clostridium proprionicum Clostridium proprionicum Clostridium proprionicum Clostridium sporogenes Archaeoglobus fulgidus	plasmalogen synthase (CPE1194) plasmalogen synthase (CPE1194) benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase benzoyl-CoA reductase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxyglutaryl-CoA dehydratase (R)-2-hydroxysocaproyl-CoA dehydratase (R)-2-hydroxyisocaproyl-CoA dehydratase (R)-2-lactoyl-CoA dehydratase (R)-2-lactoyl-CoA dehydratase (R)-phenyllactyl-CoA dehydratase (R)-phenyllactyl-CoA dehydratase (R)-phenyllactyl-CoA dehydratase (R)-phenyllactyl-CoA dehydratase	PlsR (Red1) PlsR (Red2) BcrC BcrB BcrB BcrC BcrB BzdN BzdO BzdN BzdO HgdA HgdB HgdA HgdB HadB HadC LcdB FldB FldC HgdA	$ \begin{array}{r} 41\\ 48\\ 44\\ 50\\ 44\\ 49\\ 43\\ 51\\ 45\\ 49\\ 50\\ 42\\ 54\\ 42\\ 46\\ 42\\ 46\\ 42\\ 47\\ 42\\ 46\\ 43\\ 45\\ \end{array} $	reduction/dehydration reduction reduction reduction reduction reduction reduction reduction reduction dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration dehydration	WP 025647548 WP 025647548 WP 013503791 WP 013503790 WP 107220517 WP 107220517 WP 050415404 WP 050418279 WP 148212195 WP 048086821 WP 005909813 WP 005909812 WP 012939152 WP 012939151 WP 009888224 WP 009888224 WP 009888224 WP 009888224 WP 006048111 WP 066048111 WP 066048111 WP 066048114 WP 003492352 WP 003492354 WP 003492354	100.0 19.8 19.9 20.9 19.4 19.8 21.6 17.7 21.0 20.1 19.3 22.9 21.0 21.5 22.0 20.0 19.2 19.2 19.2 19.2 20.0 21.2	19.8           100.0           18.3           23.1           20.5           21.5           18.9           17.3           20.1           19.3           19.3           20.1           19.3           19.9           21.5           18.0           20.1           21.5           18.0           20.1           21.5           20.1           21.5           20.1           21.5           20.1           21.5           20.1           21.5           20.1           21.5           20.1           22.1           23.5           20.8           24.3           22.3

 Table S2: Comparison of PIsA domains and PIsR to known HAD/BCR proteins.



**Figure S1**. A) Plasmalogen biosynthesis in animals. B) Plasmalogen biosynthesis in the aerobic bacterium *Myxococcus xanthus*. C) Plasmalogen biosynthesis in anaerobes (proposed).



Figure S2. A) Schematic for FAME/DMA lipids analysis of in bacterial pellets. Plasmalogens contain an ester linked fatty acid, which is converted to a FAME, and a vinyl-ether linked fatty acid which is converted to a DMA. In the case of diacyl lipids (not shown), both ester linked fatty acids would be converted to FAMEs. For the general structure of a bacterial plasmalogen, the R group is typically a phosphate-containing head group or a sugar. B) Results of *C. perfringens* Tn screen showing two distinct plasmalogen deficient colonies and isolation of these Tn mutants (Tn48 and Tn54) verified by re-streaking and re-staining with Schiff stain. C) GC-MS chromatogram of FAMEs/DMAs from C. perfringens HN13 WT pellet lipids showing major peaks for saturated FAMEs (C12, C14, C16, and C18) as major products, and the corresponding DMAs (C14, C16, and C18). D) GC-MS chromatogram of FAMEs/DMAs from the C. perfringens HN13 transposon mutants Tn48 (top) and Tn54 (bottom) pellet lipids showing major peaks for saturated FAMEs (C12, C14, C16, and C18) as major products, and the absence of any corresponding DMAs.





**Figure S3**. A) PCR verification of *CPE1194/1195 (\DeltaPlsA PlsR*) deletion. B) Isolation of *C. perfringens* HN13  $\Delta$ PlsA PlsR deletion mutants by Schiff staining. C) GC-MS chromatogram of FAMEs/DMAs from WT *C. perfringens* HN13 pellet lipids showing major peaks for saturated FAMEs (C12, C14, C16, and C18) as major products, and the corresponding DMAs (C14, C16, and C18). D) GC-MS chromatogram of FAMEs/DMAs from the *C. perfringens* HN13  $\Delta$ PlsA PlsR deletion mutant pellet lipids showing major peaks for saturated FAMEs (C12, C14, C16, and C18) as major peaks for saturated FAMEs (C12, C14, C16, and C18) as major peaks for saturated FAMEs (C12, C14, C16, and C18) as major peaks for saturated FAMEs (C12, C14, C16, and C18) as major products, and the absence of any corresponding DMAs.



**Figure S4**. A) A schematic representation the pET28a-based expression vector for *C. perfringens* CPE1194/1195 operon. B) Pellets from 5 ml cultures *E. coli* BL21(DE3) harboring pPIsCP with IPTG induction grown aerobically (top) and anaerobically (bottom) after staining with Schiff. C) GC-MS analysis of *E. coli* BL21(DE3) harboring pET28a (empty) showing only FAMEs and no DMAs, the most abundant peak is saturated C16-FAME. D) GC-MS analysis of *E. coli* BL21(DE3) harboring pEI28a (empty) showing only FAMEs and no DMAs, the most abundant peak is saturated C16-FAME. D) GC-MS analysis of *E. coli* BL21(DE3) harboring pEI28a (empty) showing only FAMEs and no DMAs, the most abundant peak is saturated C16-FAME. D) GC-MS analysis of *E. coli* BL21(DE3) harboring pPIsCP showing similar FAMEs and an additional peak corresponding to C16-DMA.



**Figure S5**. A) A typical HAD gene cluster and proposed enzyme architecture. B) HAD reaction mechanism. C) A typical BCR gene cluster and proposed enzyme architecture. D) BCR reaction mechanism. E) Different genetic architectures of the *pls* operon and proposed enzyme architecture. F) The plasmalogen synthase reaction mechanism based on the current BCR model involving two sequential single electron reductions, followed by protonation and elimination of water to yield a cis enol ether.



**Figure S6**. Predicted domain analysis of *CPE1194/1195* using NCBI and InterPro. *CPE1195* (*plsA*) is composed of two CoA-activase domains (Act<sub>1</sub> and Act<sub>2</sub>, component A) and one reduction/dehydration domain (Red<sub>1</sub>, component D). *CPE1194* (*plsR*) is composed of a single reduction/dehydration domain (Red<sub>2</sub>, component D).

Δ			1	2	3	4	5	6		
~	1:	A_fulgidus_HgdC	100.00	49.00	49.80	45.42	46.22	45.82		
	2:	F_nucleatum_HgdC	49.00	100.00	63.32	54.41	57.20	54.83		
	3:	A fermentas HgdC	49.80	63.32	100.00	53.70	56.76	57.98		
	4:	C_difficile_HadI	45.42	54.41	53.70	100.00	57.85	55.98		
	5:	C_sporogenes_FldI	46.22	57.20	56.76	57.85	100.00	55.98		
	6:	C_proprionicum_LcdC	45.82	54.83	57.98	55.98	55.98	100.00		
R			1	2	3	4	5	6	7	8
D	1:	R palustris BcrD	100.00	71.12	25.00	23.88	30.12	28.24	30.92	28.46
	2:	T_aromatica_BcrD	71.12	100.00	27.99	25.00	32.05	29.77	32.06	26.48
	3:	R_palustris_BcrA	25.00	27.99	100.00	69.50	33.68	31.47	24.25	23.85
	4:	T_aromatica_BcrA	23.88	25.00	69.50	100.00	33.90	32.17	25.09	26.64
	5:	Azoarcus_CIB_BzdQ	30.12	32.05	33.68	33.90	100.00	60.07	30.74	28.63
	6:	F_placidus_BzdQ	28.24	29.77	31.47	32.17	60.07	100.00	37.21	32.81
	7:	Azoarcus_CIB_BzdP	30.92	32.06	24.25	25.09	30.74	37.21	100.00	43.02
	8:	F_placidus_BzdP	28.46	26.48	23.85	26.64	28.63	32.81	43.02	100.00

**Figure S7.** A) Sequence identify matrix of HAD activation domains. B) Sequence identify matrix of BCR activation domains.

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S	HgdC_A_fermentens	123	MNDK	CZ	AA	GΤ	GR	FΙ	D 7	VM.	ΑN	ΙL	ιEΙ	/K	VS	DΙ	ΑE	L	βA	КS	. ТК	R١	7 A	ΙS	ST	СТ	VF	ΓA
Q	FldI_C_sporogenes	124	MNDK	CZ	AA	GΤ	GR	FΙ	D I	JΜ	SR	VI	ΕN	7Ρľ	<b>V</b> D	ΕL	GK	ΚĮ	L	ΕS	.KN	ΡC	СТ	ΙS	ST	CT	VF	? A
∎	HadI_C_difficile	121	MNDK	CP	AA	GΤ	GR	FΙ	L D 1	VM.	ΑK	II	ΕN	/D	VS	ΕL	GS	IS	M	ΝS	.QN	ΕV	7 S	ΙS	ST	СT	VF	? A
<u> </u>	HgdC_F_nucleatum	125	MNDK	C	A A	GΤ	GR	FΙ	D I	JΙ.	ΑK	VL	ιE	7 N :	LΕ	DΙ	ΕK	ΙL	ΡE	КS	. T V	D١	7 A	ΙS	ST	CT	VF	? A
	BzdP_Azoarcus_CIB	131	GNEK	C	AA	GA	GS	FΖ	ΑEΖ	ΑM	SR	ΑL	JQ I	S	LΚ	ΕF	GE	AS	5 L	RS	.DK	S I	ΓP	ΜN	ΑQ	CT	VF	ΓA
	BzdP_F_placidus	121	LADK	CP	AA	GA	GS	F٦	7 D 7	A M	VR	ΥL	ιE	/ T ]	ΡE	ΕF	GF	. L 7	ΥL	QΑ	. ТК	S I	ΕP	ΜN	ΑQ	CV	IF	? A
S	BzdQ_Azoarcus_CIB	158	MNDK	CP	AA	GΤ	GR	GN	٩E	ΙI	SD	LM	1 Q ]	[P]	ΙA	ΕL	GP	RS	SF	DVI	ΞТΕ	ΡE	ΞA	VS	SI	CV	VF	ΓA
R C	BzdQ_F_placidus	156	MNDK	C	AA	GΤ	GR	G ]	ΓE	νV	CD	LL	ΙAΙ	7 P [	ΙΤ	ΕA	. <b>G</b> E	$\mathbf{L}$	Γ	K V I	ΟEΕ	ΡE	ΞP	VS	ТТ	CV	VF	? A
No	BcrA_R_palustris	294	MNDR	CZ	A A	GC	GR	ΥI	G	YI.	AD	ΕM	INN	1G	LΗ	ΕL	GP	LΖ	١М	QΣ	.NR	ΡÆ	ΑR	ΙN	ST	CT	VF	? A
	BcrA_T_aromatica	294	MNDR	CP	AA	GΤ	GR	ΥI	- G 1	YV.	AD	ΕM	INN	1G	LΗ	ΕL	GP	LΖ	ΔM	ΚS	. ТК	S I	[ R	ΙN	ST	CT	VF	ΓA
	BcrD_T_aromatica	126	MTSQ	C	A S 🤇	GS	GQ	FΙ	ΓΕl	II.	AR	ΥL	G ]	ΕA	ΩD	ΕI	GS	$\mathbb{L}^{S}$	SΤ	QΑ	.DN	ΡE	ΞV	VS	SI	CA	V I	ΓA
	BcrD_R_palustris	126	MTSQ	C	S C	GS	GQ	FΙ	ΓEΙ	II.	AR	ΥL	G ]	ΓA	ΩD	ΕI	GS	$\mathbb{L}^{S}$	ŞQ	RΑ	.DN	ΡE	ΞK	VS	GΙ	CA	V I	ΓA
	PlsA_Act1_C_perfringens	119	MNSS	C	A G 🤇	GΤ	GA	F ]	ΕD	2 <mark>M</mark> .	ΑH	LM	IGI	T	VD	ΕM	[ D N	LS	Γ	ΚH	.EN	ΙŊ	ζE	ΙA	SR	CG	VF	ΓA
	PlsA Act2 C perfringens	439	LNEA	CS	SS	GC	GS	F 1	ΕES	SF.	AN	QM	IG ]	[D]	VΕ	ΤF	SK	L	βL	FΑ	. КН	GP	4 D	LG	SR	ΞT	VF	ΡM
				▲																					1	▲ .		

**Figure S8**. Protein sequence alignment of activation domains from HADs, BCRs, and PIsA (Act<sub>1</sub>, AAs 1-288) and PIsA (Act<sub>2</sub>, AAs 289-620) showing conserved cysteines for iron-sulfur cluster ligation.



**Figure S9**. A) GC-MS analysis of *E. faecalis* grown aerobically showing only FAMEs present. B) GC-MS analysis of *E. faecalis* grown anaerobically showing FAMEs and plasmalogen-derived DMAs.



**Figure S10**: A) *E. coli* expression plasmid for the single gene *pls* operon from *E. faecalis* OG1RF. B) GC-MS analysis of *E. coli* BL21(DE3) harboring pET28a (empty) showing only FAMEs and no DMAs, the most abundant peak is saturated C16-FAME. C) GC-MS analysis of *E. coli* BL21(DE3) harboring pPIsEF showing similar FAMEs and an additional peak corresponding to C16-DMA.